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Sexually transmitted diseases (STDs) are among the major causes of morbidity for both men and women in the U.S. Comprehensive prevalence studies among military women for all these infections have not been performed and STD medical facilities in the Army are limited. Implementation of an STD screening and treatment intervention strategy based on new, highly sensitive and specific diagnostic tests could have a multifaceted impact on Army women and on the medical system. This study was designed to assess the sensitivity and specificity of a single self-administered intravaginal swag (SAS) for detection of gonorrhea, chlamydia, trichomonas and human papilloma virus (HPV) among active military women attending a health care facility at Fort Bragg, North Carolina. Of 793 women who volunteered for study participation, 33% were Caucasian, 59% were African American and 8% were other races; the mean age was 24 years old. The SAS compared to Standard Operating Procedure (SOP) diagnostics detected more cervical gonorrhea (5.6% vs 2.3%), more chlamydia (11.2% vs 8.9%), and more trichomonas (7.7% vs 5.1%). HPV was identified from 36.5% of specimens and higher HPV prevalence was related to greater numbers of sexual partners in the previous 6 months. In preliminary analyses, 13 cancer-associated HPV types were far more frequent in women with squamous intraepithelial lesions on Pap smears. Furthermore, we found that the SAS transported as a "dry" swab, resulted in equal STD detection compared with the SAS shipped "wet" in transport medium. This screening method is exciting since it may expedite diagnosis and treatment of women "in the field", and it may eliminate any biohazard transport or confidentiality problems and prove more cost-beneficial.

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Anne Pagels 12-15-98

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Introduction

Sexually transmitted diseases (STDs) are among the major causes of morbidity for both men and women in the U.S. Among women serious sequelae such as pelvic inflammatory disease (PID), ectopic pregnancy, infertility, and cervical carcinoma can occur as a result of undiagnosed STDs (1). The direct medical care costs of these diseases are estimated to be in excess of five billion dollars annually (2). Infection rates for STDs in young, sexually active women between the ages of 18 and 30 may range from 5 to 25% (1). Since early infection may be asymptomatic in over half of the women, many do not seek health care until the development of complications.

Comprehensive prevalence studies among **military** women for all these infections have not been performed, but a recent large-scale chlamydia screening study which we conducted through funding from the Department of Defense Women's Health Research Program demonstrated alarmingly high rates among female military recruits. The overall prevalence of chlamydial infection was 9.2%, with a peak of 12.2% among the 17-year-old recruits (3). Other small studies have demonstrated gonorrhea rates from 2 to 5%, trichomonas rates from 5 to 25%, and human papilloma virus (HPV) estimated rates ranging from 20 to 40% (4-7). These rates are substantially higher compared to 1993 Centers for Disease Control and Prevention (CDC) surveillance rates in the U.S. female population of 0.2% for chlamydia, and 0.14% for gonorrhea (8).

The diagnosis and treatment of STDs and their complications among active duty soldiers can result in substantial direct medical costs as well as disability and reduced deployability. Providing easy access to diagnosis and treatment can effectively reduce STD prevalence and incidence. However, STD medical facilities in the Army are limited. Traditional approaches to STD diagnosis among women, even when trained medical and paramedical personnel are available, require full gynecologic examination and laboratory diagnostic tests, which are often not possible at the field level. Social impediments such as ostracization, embarrassment, and insensitive provider attitudes may prevent rapid access to care and lead to further clinical complications.

The recent development of molecular STD probes has revolutionized diagnostic approaches. These new tests, based on DNA technology and extensively evaluated in our laboratories, can be self-administered, can decrease the need for physical exam, are stable for weeks after obtaining specimens at room temperature, and are highly sensitive and specific. Implementation of a STD screening and treatment intervention strategy based on these diagnostic tests could have a multifaceted impact on Army women and on the medical system.

This proposal was designed to assess the sensitivity and specificity of a self-administered intravaginal swab (SAS) for detection of these STDs among active military women attending a health care facility at Fort Bragg, North Carolina.

Background

New diagnostic assays for chlamydia and gonorrhea using molecular techniques have been developed over the past several years. In assessing sensitivity, it is generally accepted that for most organisms culture provides the best sensitivity and specificity but may be variable depending on the pathogen. In most settings, the diagnosis of gonorrhea is made most cheaply and reliably by culture. In addition, Gram stain diagnosis as presumptive evidence for gonococcal disease has a sensitivity greater than 95% in men, but is lower in women at approximately 60% (9,10).

For years, the diagnostic gold standard for chlamydia infection among both men and women was considered to be in-vitro cell culture. Chlamydia cultures, however, are expensive and may be only 85% sensitive in the best laboratories because of a variety of inherent limitations, including transport requirements, sample reliability, and infectious load (11). Gram stains of cervical and urethral discharge to detect increased numbers of polymorphonuclear cells is helpful to confirm a "syndromic diagnosis" that may be due to chlamydia, but sensitivity and specificity of Gram stains for chlamydia cervicitis is not optimal (12).

Consequently, a wide variety of more rapid and less expensive STD assays has been developed, especially for detection of chlamydia. These include enzyme immunoassays (EIAs) to detect chlamydial and gonococcal antigen in cervical secretions and urine; fluorescent monoclonal antibodies for the direct visualization of chlamydial elementary bodies on smears; rapid antigen tests designed for use in physician and STD clinics; and molecular probes including nucleic acid hybridization tests, PCR, and LCR. With the exception of PCR and LCR, all nonculture methods of antigen detection for both gonorrhea and chlamydia are much less sensitive, detecting between 10^3 and 10^4 infectious particles per milliliter per sample. Sensitivities range from 60% to 85%, and some tests may also give false-positive results. PCR and LCR, however, have been shown to detect 1 to 10 infectious particles per sample and have sensitivities above 90% with almost 100% specificity.

The commercial PCR assay (Amplicor, Chlamydia; Roche Diagnostic Systems, Branchburg, N.J.) has been evaluated by our laboratory for female and male urine specimens and found to be highly sensitive and specific in multiple studies (13,114). Published studies comparing PCR to a revised gold standard of a positive culture or another positive test including molecular amplification with different primers demonstrated sensitivities ranging from 89% (one site) to 98.6% and specificities from 99.7 to 100% (15). The one clinical site reporting a sensitivity of 89% for endocervical specimens reported that a labile inhibitor influenced the sensitivity (16). Our most recent study submitted for publication, which includes PCR results for cervical swabs as compared to culture for 418 women indicated that the sensitivity was 86% and the specificity was 99% (17). Our most recent data from a national CDC-funded prevalence study, using this assay for 5,955 female cervical specimens from STD patients, indicated an increase in prevalence from 7.8% by culture in 1993 to 13.4% by PCR in 1994-1995 (Gaydos, unpublished data). In this same prevalence study, 2861 cervical specimens from 2 family planning clinics had a positivity rate of 8.0%

Recently, self administered vaginal swabs (SAS), a novel specimen type, were evaluated by PCR for chlamydia infection. Even though chlamydial organisms infect the cervical columnar epithelial cells, the power of the amplified DNA technology (DNA from one

organism is amplified one million-fold) is sufficient to detect those infected cells, which are exfoliated into the vaginal introitus. These self-administered vaginal swabs were tested by PCR and compared to cervical PCR as well as culture as the gold standard for 26 chlamydia positive women (18). Of the infected women, cervical culture detected 21 (81%), cervical PCR detected 22 (85%), vaginal introitus self-administered detected 21 (81%), and vaginal introitus, nurse administered, detected 24 (92%). Similar recent studies reported SASs to be as sensitive as a speculum-obtained swab for PCR detection of chlamydia and gonorrhea cervical infections (19,20) and similar techniques have also been proven successful for trichomonas (21) and HPV detection (22,23).

Self-collected samples also appear to be suitable for HPV diagnosis. Morrison and co-workers (22) asked 25 women investigated at a colposcopy clinic to also provide a second cervical lavage sample collected at home with a MY-PAP kit for HPV diagnosis. Seventeen women (68%) complied with the request; 16 samples were satisfactory. 88% of the self-collected samples were positive by HPV-PCR. There was essentially complete concordance between results of home-collected samples and clinic samples. Fairley et al. (24) compared specimens collected from self-administered tampons with specimens collected at the clinic during a gynecological examination. In PCR tests of paired specimens from 48 women, 73% of cervical scrapes and 69% of tampons were HPV-positive. There was excellent correlation between HPV type-specific diagnoses in samples collected by the two methods. Moscicki (23) compared pairs of self-collected vaginal samples, obtained with dacron swabs, with samples collected during gynecological examinations. HPV was detected by non-amplification based assay. Seventy-four pairs were negative and 24 pairs were positive with identical results, in the two tests. In ten specimens which gave discordant results, the self-collected samples were as likely to be HPV-positive as the speculum-assisted samples.

Our laboratory stability studies for clinical and laboratory strains of *C. trachomatis* have been performed and indicate that the chlamydial DNA is stable for 14 days in the commercial Roche molecular Systems Amplicor, Chlamydia transporter. A positive patient sample was aliquoted in 11 tubes and two series of 11 transporter tubes were inoculated with 5 and 50 inclusion forming units (IFU) of *C. trachomatis* serovar E per tube. These tubes were set out at room temperature to simulate transport conditions. These transporter tubes contained sodium dodecyl sulfate, 0.4% (SDS) and TRIS buffer, 0.13%, so that potential nucleases which might degrade DNA would be expected to be inhibited. At days 0 to 14, specimens were removed from the room temperature rack and processed according to manufacture's directions: one millileter of treatment buffer (Tween 20), was added to the specimen to neutralize the SDS. The specimens were then refrigerated until the next PCR batch was performed (2-4 days). All of the room temperature stored tubes gave positive tests in the subsequent PCR tests, indicating that mailed or transported chlamydia-containing specimens would be stable for up to 14 days and could be detected as positive by PCR of the chlamydial DNA. In addition, laboratory studies from the Roche Molecular Systems using real patient specimens have indicated that specimens kept at room temperature for up to 33 days, still remained positive in the PCR test, as compared to culture. An HPV-positive clinical specimen was employed to test the stability of HPV DNA in the transport medium in a way similar to that described above for chlamydia DNA. The HPV hybridization signal after PCR did not decrease in intensity through the observation period of 8 days.

Our laboratory stability studies for a fresh clinical isolate of gonorrhea indicate that the DNA from this isolate is stabile in the Roche transporter for 11 days at room temperature.

The transporter was inoculated with 10 organisms, as determined by plating serial 10-fold dilutions of a # 0.5 Barium Sulfate standard (10^8 organisms/ml) and actual plate counts of the diluted specimens. The inoculated transporters were treated as above by incubating the tubes at room temperature and processing one per day. The stored and processed specimens were tested in the Roche multiplex Chlamydia/gonorrhea PCR test. The DNA remained positive in the PCR test for the maximum days tested (11) with no difference in optical density from day 0 (1.019) to day 11 (1.256). The test was positive for 100 organisms for this time period, but not for 1 organism.

Our laboratory has also conducted stability studies for a clinical specimen from a woman shown to be positive for trichomonas by wet mount preparation in a manner similar to that described above. Fifty microliters of the clinical swab specimen were added to the Roche transporter tubes, which were incubated at room temperature. Each day, from day 0 to day 9, one transporter was processed according to manufacturer's directions, which consisted of adding 1 milliliter of treatment buffer (Tween 20). Treated specimens were used for our in-house trichomonas PCR, and they were shown test positive, by giving the expected two bands on a silver stained polyacrylamide gel. Dilutional studies, using cytometer-counted trichomonas organisms, were shown to be able to detect both 50 and 5 trichomonas organisms per PCR. The trichomonas organism can also be detected in the multiplex system by adding trichomonas primers to the multiplex PCR mixture used for the chlamydia-gonorrhea assay. Further study may allow PCR multiplexing of all three organisms in the future, which could decrease the overall PCR testing protocol. More testing and development is needed before attempting this on any clinical specimen, but this approach deserves further study.

These SAS and specimen stability data indicate that this approach, namely a non-invasive, self-administered swab specimen for STD detection, has the potential to drastically improve STD screening in military women. During the 2 years of this study, we implemented and tested this hypothesis. We examined the sensitivity and specificity of the SAS compared to Standard Operating Procedures (SOP) at Fort Bragg Epidemiology and Disease Control Clinic. During the first collection period, we tested their performance when they were collected by the study clinician, then we determined their performance when collected by the patient herself. Overall, women found the SAS acceptable with little complaints regarding the self-administered method. In general, the SAS detected at least as many, if not more, STDs than did SOP. We are still confirming if positive test SAS results reflect true infections, and we will continue to analyze the relevance of trichomonas detected by PCR techniques as well as HPV PCR results.

Nonetheless, the results from this 2 year study show that patient administered swabs can be used for accurately diagnosing STDs and that for gonorrhea and chlamydia infections, the specimen can be shipped to the laboratory in a "dry" condition. These are important data. Use of SAS diagnostics for all common STDs could revolutionize the approach to efficient and cost effective STD testing for military women in an effort to reduce the morbidity associated with these diseases. Not only could access to medical care through collecting and sending the specimen to a medical facility by mail or courier be increased, but compliance to submit to STD screening may be enhanced greatly, since a speculum examination of the vagina and cervix could be avoided, at least for the screening test.

Technical objectives were to:

1. Establish prevalence of the above STDs among consenting active duty military women who present to the Fort Bragg Epidemiology and Disease Control (EDC) clinic for gynecological evaluation.
2. Determine sensitivity and specificity of **clinician-administered** SASs to detect the above STDs using PCR techniques compared to that obtained by standard diagnostic tests performed on specimens obtained during speculum-assisted gynecologic examination. The techniques include:
   - Gonorrhea: Culture
   - Chlamydia: Enzyme Immunoassay Test
   - Trichomonas: Light Microscopy exam of normal saline reparation of vaginal secretions (wet prep)
   - HPV: Papanicolaou smear (Pap smear)
3. Determine sensitivity and specificity of the clinician-administered SASs compared to additional "gold standard" tests performed on specimens obtained during standard speculum-assisted gynecologic examination. These additional techniques include:
   - Chlamydia: PCR on endocervical secretions when discrepancy between SAS and EIA occur;
   - Trichomonas: Culture
   - HPV: PCR of cervical scrape
4. Determine the clinical profile of women with the above STDs who present to the EDC by administrating a confidential questionnaire.
5. Determine the performance ease of patient-administered SAS among a subgroup of 275 women attending the EDC and consenting to study participation. Modify the collection kit and self-use instructions to improve utility.

Methods

Study Population: The Epidemiology and Disease Control (EDC) Clinic at Fort Bragg is a fully equipped medical treatment facility. Average patient census for active duty females presenting with vaginal discharge and/or potential STD exposure has been approximately 100/month for the past 2.5 years. Using clinic-standard diagnostic tests (modified Thayer-Martin culture for *N. gonorrhoeae*, Syva Microtrak EIA for *C. trachomatis*, and wet prep for *T. vaginalis*), 20% of these active duty females are confirmed to be infected with one or more of these STDs. True incidence of these infections is unquestionably higher due to the relative insensitivity of the tests available for routine patient care in this facility, but the estimated prevalence rates based on 1995 statistics are 5% of gonorrhea, 14% for chlamydia, and 3% for trichomoniasis. Tools for laboratory diagnosis of human papillomavirus infection are not available, but Pap smears are performed in this clinic on study volunteers for the duration of this study.

Fort Bragg EDC Procedure: During this study, consecutive active duty military women, 18 to 59 years of age, attending the EDC clinic were invited to participate in the study. This clinic evaluates walk-in or referred patients who present with genitourinary symptoms, are known contacts to an individual with a diagnosed STD, or who present for routine STD screening because of self-perceived risk. Any woman who presented to the EDC for these complaints was approached for study enrollment. Women were approached for enrollment by the study assistant during the time between registration and clinician evaluation so as not to interfere with regular clinic flow. All study information, as well as informed consent, was relayed to the patient in a separate clinic room and in a confidential manner.

If the woman agreed to participate and signed the informed consent, she was assigned a **unique, confidential study number, which was used on all questionnaires and study specimens**. The study clinician administered a 10 minute standardized questionnaire. From our experience with previous studies, we have found that trained, gender-matched clinicians establish more open relationships with patients, and reduce patient reporting biases. We trained a study assistant to administer the questionnaire in a "standardized" fashion, without leading the patient or deviating from the printed word in any significant fashion. The brief questionnaire was developed to collect data regarding demographics, menstrual and reproductive history, contraceptive practices, use of female hygiene products, access-to-care patterns, frequency and results of previous Papanicolaou (Pap) smears. Data regarding reason for visit, symptom type and duration, and antibiotic use during the previous two weeks was recorded. Information was collected to determine risk factors related to STDs and HIV, such as sexual orientation, numbers and types (casual, regular, anonymous) of sex partners and sexual practices (e.g. oral-genital, genital-genital, rectal-genital) in the previous year, complete history of prior STDs or gynecologic infection, drug or alcohol use pattern relative to sexual activity, and drug and alcohol use pattern among sexual partners. The questionnaire was reviewed by investigators on an on-going basis to assure the integrity of the data. These data will be analyzed to identify behavior profiles which put military women at risk for STD acquisition and thus to further develop and refine educational and instructional information regarding the use of the SAS.

After questionnaire completion, all participants received a standard pelvic exam. Prior to starting the pelvic examination, **one vaginal swab** was collected from each participant by the study clinician prior to insertion of the speculum. The study clinician initially administer

the SAS in order to determine the best technique and timing to obtain optimal SAS sensitivity and specificity performance, and thus determine procedures for patient self-insertion. The swab was sent to the Johns Hopkins STD Research Laboratory for PCR for *C. trachomatis, N. gonorrhoeae, T. vaginalis*, and HPV. Following this specimen collection, the speculum was inserted and routine diagnostic tests for these and other infectious agents were obtained according to SOP. Vaginal swabs were obtained for trichomonas culture, pH testing, wet preparation for bacterial vaginosis (BV) and trichomonad detection, and 10% potassium hydroxide (KOH) preparation for yeast detection. Cervical swabs were obtained for chlamydia enzyme immunosorbant assay testing (EIA), gonorrhea culture, chlamydia PCR, HPV PCR and cervical Gram's stain. A cervical Pap smear was obtained to detect changes that may be consistent with HPV infection (e.g. koilocytosis).

The sensitivities of vaginally obtained swabs were calculated using the results from the standard collection method obtained by use of speculum. For specimens that were positive by the vaginal swab and negative by standard culture, FDA-approved PCR assays will be performed on the speculum-obtained swabs, which were saved to help resolve any discrepancies. Treatment of chlamydia, gonorrhea, and trichomonas was based on the gold standard FDA licensed tests, followed the SOP algorithms, and was in accordance to CDC STD treatment guidelines (25). Table 1 summarizes these procedures:

Table 1: Comparison of Procedures

Procedure	Number Swabs Collected	Test Performed
Self- Administered Swab	One Intravaginal Swab	PCR for gonorrhea PCR for chlamydia PCR for trichomonas PCR for HPV
Standard Operating Procedures	Three Vaginal Swabs Two Endocervical Swabs	Wet Prep for Trichomonas and BV Swab for vaginal pH test 10% KOH prep for yeast Gram stain & Culture* for gonorrhea EIA test for chlamydia*
Added EDC Procedure	One Vaginal Swab	Trichomonas Pouch Culture*
Johns Hopkins Added Procedure	One Endocervical Swabs One Exocervical Scrape	PCR for chlamydia in case of discrepants* PCR for HPV*

***indicated accepted Gold Standard for disease diagnosis**

Toward the completion of approximately 450 SAS, we conducted the second half of the study to determine the performance and utility of the SAS among the EDC women. The

study assistant gave a swab collection kit to each consenting woman. The patient was instructed to go to the ladies room and collect the intravaginal swab before seeing the clinician. Upon presenting the collection kit to the clinician, the patient was asked to critique the educational and instructional message, as well as the utility and ease of swab collection.

Laboratory Procedures

Neisseria gonorrhoeae and Chlamydia trachomatis: This combination test (Roche Molecular Systems, Branchburg, N.J.) utilizes polymerase chain reaction (PCR) combined with DNA probe hybridization in an ELISA-like detection assay. The target sequence for DNA amplification for *C. trachomatis* is the cryptic plasmid, which is common to all strains and not found in any other bacteria. *N. gonorrhoeae* contains DNA methyl transferase gene M:NgoP11, and a region of this gene specific to only *N.gonorrhoeae* is the target sequences will be performed in a 96-well format using a Perkin Elmer Cetus thermocycler. Following amplification, two detection assays, one for *C. trachomatis* and one for *N. gonorrhoeae*, will be performed. This procedure allows for the detection of either, both or neither organism. Sensitivity and specificity as compared to culture have been reported to be 96.1% and 100% for *C. trachomatis* and 94.3% and 97.8% for *N. gonorrhoeae*, respectively. The gonorrhea PCR results will be compared to the gonorrhea culture as gold standard; the chlamydia PCR will be compared to the Syva EIA, and cervical PCR will be used to resolve any discrepancies between SAS PCR and Syva EIA for chlamydia.

For this procedure, chlamydial or gonococcal target DNA is amplified by the use of two sets of biotinylated oligonucleotide primers, complementary to regions of DNA and polymerase to extend the 5'-3' synthesis of DNA, using excess deoxynucleoside triphosphates from the reaction mixture. Successive cycles amplify the DNA 2^{30} times and creating an amplicon. Oligonucleotide probes, specific to the amplicon, and which are bound to a microwell pate, capture the DNA. The subsequent detection assay utilizes avidin-horseradish peroxidase conjugate which binds to the plate-captured amplicon. The substrate is tetramethylbenzidine-peroxide. A color reaction develops which is measured in a microplate spectrophotometer. Positive and negative control will be used for every batch. Use of an internal control will serve to rule out the presence of inhibitors.
PCR was performed on swab specimens by using a prototype, rapid, nonradioactive PCR-based assay (Roche Molecular Systems, Branchburg, NJ) according to investigational test procedures. A100- uL sample of each chlamydia 2-sucrose-phosphate transport medium (STM) was diluted with an equal volume of AMPLICOR CT/NG lysis buffer. The resulting lysed specimen was then mixed with 200 uL of AMPLICOR CT/NG specimen diluent.

Fifty microliters of the processed patient sample or control was then added to each PCR tube containing 50 uL of the PCR master mix as described previously (27). PCR amplification was carried out for 35 cycles with the GeneAMP 9600 thermocycler (Perkin-Elmer Cetus, Norwalk, Conn). After amplification, the PCR products were detected as previously described (27). Optical density of the reaction mix was then measured using a spectrophotometer at 450 nm. Values greater than or equal to 0.8 were considered positive; those less than 0.2 were considered negative. Specimens with optical density values between 0.2 and 0.8 were retested in duplicate. If two of the three test results (original and duplicate repeats) were found to be $\geq$0.25, the sample was considered positive for chlamydia or gonoccocal DNA by PCR. If two of the three test results (original and

duplicate repeats) were found to be <0.25, the sample was considered negative for chlamydia or gonococcal DNA by PCR. Negative controls were required to have an optical density value of <0.2, and the positive control had to have an optical density of ≥2.0 for the test to be considered valid.

Analysis of discrepant results. For *C. trachomatis* specimens that were PCR positive and EIA negative, or PCR negative and EIA positive, MOMP-based PCR was performed on the STM. For *N. gonorrhoeae* specimens that were PCR positive and culture negative, LCR (Abbott Diagnostics, Abbott Park, IL) was performed on the dry swab specimens.

Trichomonis vaginalis: Materials and methods for the identification of *T. vaginalis* by culture and PCR are described in detail in our manuscript, which appears in the Appendix.

Human papilloma virus:
HPV diagnosis by PCR: PCR were performed on cervical swabs (gold standard) and on vaginal swabs. Pap smears served as the surrogate SOP for HPV detection. The PCR technology used in our laboratory has been described in detail (). We tested the specimens for HPVs with MY09/MY11 L1 consensus primers, which amplify a large number of HPVs. A segment of the ß-globin gene was co-amplified in the same tube with a set of ß-globin primers. The PCR products were identified in dot blot hybridization format with biotin-labeled HPV generic probe, 26 HPV type-specific probes and ß-globin probe. The specimens were classified as positive for specific type(s), positive only by generic probe and negative. Specimens negative for both ß-globin and HPV amplification were categorized as "unsatisfactory". The strength of the hybridization signal was graded 1+ to 4+, with 4+ being the most intense signal. Viruses were grouped into three categories: strong cancer risk: HPV-16, 18, 45 and 31; moderate cancer risk: HPV-33, 35, 39, 51, 52, 56, 58, 59, 68; low cancer risk: all other HPV types.

Risk Factor Analysis. Categorical variables will be examined using Pearson's chi square test (Fisher exact test will be used when cell size is <5). Multivariate logistical regression analysis will be performed using the Statistical Application Software or True Epistat computer program. On the basis of univariate analysis, the most predictive factors will be used for the regression analysis. Separate analyses will be done for each STD and then for the presence of any STD. A stepwise backward elimination process will be used to arrive at the final model of important risks for infection(s). The sensitivity of a risk factor will be defined as the probability of the factor being present for all patients with a positive test for infection. The positive predictive value for a risk factor will be the proportion of positive tests among all women who have the risk factor.

The model will be used to predict which combination of risk factors will give the highest sensitivity and specificity for finding women who would test positive for infection, as well as have a high positive predictive value. We will construct separate models using SAS results and SOP test results to indicate the presence of the STD(s) and compare the risks identified.

.

Results

Analysis is ongoing on the final data set. We have completed preliminary analysis regarding

risk behaviors and STDs as diagnosed by SOP results. Analyses of risk factors related to SAS results and comparisons between these two diagnostic methods are still ongoing. We present data analyzed to date.

A total of 793 women enrolled in this study over a 20-month period from March 17 1997 through November 24, 1998. Of these 793 women, 258 (33%) were Caucasian, 474 (59%) were African American; and 61 (8%) were of other races. According to SOP diagnostic procedures, of the 793 women in the study 790 had chlamydia EIA done: 717 were negative and 73 (9%) were positive. Gonorrhea was detected from cervical cultures among 17 of 786 women tested, and trichomonas was diagnosed by wet preparation exam in 22 of 793 women. Trich pouch cultures were positive among 39 of 791 women. Table 2 presents STD detection according to SOP by race.

Table 2: STDs detected according to SOP stratified by Race

Race	Caucasian (N =258)	African American (N=474)	Other (N =61)
Gonorrhea by culture (N= 17)	2 (1%)	14 (3%)	1 (2%)
Chlamydia by EIA (N = 73)	25 (10%)	40 (8%)	8 (13%)
Trichomonas by Trich Pouch Culture (N = 39)	6 (2%)	33 (7%)	0

The median age of women in the study was 25.06 years of age (mean 24). The major reason for visit was symptom evaluation (655/793, 83%), with 20% reporting for STD screening and 7% reporting for treatment as a known contact to someone with a STD. Marital status by STD is shown in Table 3.

Table 3: SOP Diagnoses by Marital Status

Marital Status	Single (N= 433)	Married (N = 209)	Divorced (N = 40)
Gonorrhea by culture (N= 17)	12 (3%)	3 (1%)	1 (1%)
Chlamydia by EIA (N = 73)	50 (12%)	11 (5%)	6 (6%)
Trichomonas by Trich Pouch Culture (N = 39)	23 (5%)	12 (6%)	3 (3%)

Over half of the women in this study reported a prior STD history (52%): 8% reported a

prior history of gonorrhea; 28% chlamydia; 7% trichomonas; 9% genital herpes; 1% bacterial vaginosis; 1% pelvic inflammatory disease; and 8% genital warts. A new partner was reported for 54 0f 793 (7%) and the average number of sexual partners in the previous 6 months was 1.37 (median 1, range 0-20). Of 793, 113 reported always using condoms; 345 (44%) sometimes, and 325 (41%) never using condoms, and 7 were not recorded. However, among those self-reported condom use categories, STDs were diagnosed as presented in Table 4.

Table 4: SOP Diagnoses by Self-reported Condom Use

Condom Use	Always (N = 113)	Sometimes (N=345)	Never (N = 325)
Gonorrhea by culture (N= 17)	1 (1%)	10 (3%)	6 (2%)
Chlamydia by EIA (N = 73)	9 (8%)	39 (11%)	24 (7%)
Trichomonas by Trich Pouch Culture (N = 39)	4 (4%)	17 (5%)	18 (6%)

Almost half of the women (386/793) reported no contraceptive use (49%), other than condoms. The most common type of contraception reported was oral contraceptive pills (257 of 793, 33%), and 65 women (8%) reported tubal ligation/hysterectomy.

Of the 73 women with chlamydia diagnosed by EIA, 44 were treated at the day of their initial visit primarily for contact to chlamydia. For three women, no information regarding treatment was noted, and of the 26 women treated after presentation, the average number of day until treatment was 7.22 (median 8, range 2 – 17 days). For women diagnosed to have gonorrhea by culture, 9 were treated at presentation and 8 were treated on an average of 4.38 days later (median 4 days, range 3 – 8 days). For trichomonas infections, 30 women were treated at presentation, 8 received treatment on an average of 5.2 days later (median 3, range 1- 19 days) and 4 had no record of treatment.

Results: ***Neisseria gonorrhoeae and Chlamydia trachomatis:***
To date, of the 793 specimens available for testing, **matched specimen** results from Ft. Bragg (Standard of Care) and Johns Hopkins (Research Vaginal Specimens) are available for 694 specimens: 453 collected by the study clinician and 243 self collected by the patient. Table 5 presents prevalence rates of gonorrhea and chlamydia by SOP versus SAS for the total specimens collected.

Table 5: STD Prevalence by Diagnostic Method: SOP versus SAS

Organism	Site	Total	Positive	Prevalence
Gonorrhea	Ft. Bragg (SOP)	694	16	2.3%
	JHU (SAS)	694	39	5.6%
Chlamydia	Ft. Bragg (SOP)	694	62	8.9%
	JHU (SAS)	694	78	11.2%

Discrepant Specimens:

For gonorrhea: Ft Bragg positive/ JHU negative: 1
JHU positive / Ft. Bragg negative: 24.

For chlamydia: Ft. Bragg positive/ JHU negative:
JHU positive/ Fort Bragg negative:

Specimens are currently being analyzed according to procedures stated previously.

Results are available for specimens run in the "wet" STM transport medium versus those shipped "dry" and then reconstituted as described in the methods section.

Table 6: Prevalence of Gonorrhea and Chlamydia by PCR according to Transported Specimen State: Wet versus Dry

Organism	Swab type	Total	Positive	Prevalence
Gonorrhea	"Wet"	687	39	5.7%
	"Dry"	689	39	5.7%
Chlamydia	"Wet"	691	78	11.3%
	"Dry"	693	72	10.4%

We have also done preliminary analysis regarding performance of the SAS as collected by the clinician compared with SOP (Methods 1) and the SAS as collected by the patient herself compared with SOP. These data are presented in Table 7. Isolation rates in Table 7 represent that obtained by PCR of wet specimens for gonorrhea and chlamydia and culture for trichomonas. These results are not adjusted for discrepant positives and these will be considered when completed.

Table 7: Prevalence of STD from Clinician versus Patient Collected SAS

Organism	Swab type	Total	Positive	Prevalence
Gonorrhea	Method 1 (Clinician)	446	24	5.4%
	Method 2 (Patient)	241	15	6.2%
Chlamydia	Method 1	450	52	11.5%
	Method 2	241	26	10.8%

Trichomonas vaginalis

A PCR test for the detection of *T. vaginalis* using vaginal swab samples was developed to incorporate the molecular detection of *T. vaginalis* to the growing list of STDs that can be detected by DNA amplification techniques. A primer set BTUB 9/2 was designed to target a well-conserved region in the beta-tubulin genes of *T. vaginalis*. All strains (15/15) strains of *T. vaginalis* tested were successfully detected by PCR giving a single predicted product of 112 base pairs (bp) in gel electrophoresis. No such targeted product was amplified with DNA from *T. tenax, T. gallinae, Chlamydia trachomatis, Neisseria gonorrhoeae, Giardia lamblia, Chilomastix sulcatus Dientamoeba fragilis,* and *Entamoeba histolytica.* An optimal analytical sensitivity of one *T. vaginalis* organism per PCR reaction was achieved. Culture, performed with the Inpouch TV culture system, was examined daily using a light microscope to identify *T. vaginalis*. Twenty-three of 350 (6.6%) vaginal swab samples from women attending an army medical clinic were culture positive for *T. vaginalis*. Of these culture positive specimens, PCR detected 22 of 23

(96%) using primer set BTUB 9/2, and wet preparation detected only 12 of 23 (52%). Seventeen specimens were BTUB 9/2 PCR positive and culture negative. Ten of these discordant specimens were adjudicated as true positive by PCR using primer sets TVA 5-1/6 and/or AP65 A/B, which target different regions in the T., *vaginalis* genome, and seven were adjudicated as false positive. The sensitivity of BTUB 9/2 PCR was 97% and the specificity 98%. The sensitivities of culture and wet preparation were 70% and 36%, respectively. The diagnosis of *T. vaginalis* infection by PCR is a sensitive and specific method that could be incorporated into a joint strategy for the screening of multiple STDs using molecular amplification methods. Complete details of methods and results appear in our manuscript included in the Appendix section.

To date 766 specimens have been tested for *T.vaginalis* by PCR. The results appear in Table 8.

Table 8: Prevalence of Trichomonas detected by culture and PCR

Organism	Method	Total	Positive	Prevalence
Trichomonas	Culture	766	39	5.1%
	PCR	766	59	7.7%

Discreptants for this number of specimens included 4 culture positive/PCR negative samples and 24 PCR positive/culture negative samples. Of these 7 were confirmed as true positive and the adjusted prevalence was 52 positive of 766 (6.8%).

Human papilloma virus results:

HPV infections are etiologically linked to invasive cervical cancer and to preinvasive squamous intraepithelial lesions (SILs) of the cervix. HPV diagnoses may therefore be useful in the identification and management of cervical neoplasia. Identification of many sexually transmitted infections from a single swab has obvious advantages, especially for military women who may be stationed in different parts of the world and have variable access to health care.

Treatment of specimens for efficient HPV diagnosis: At the beginning of the study, we found that specimens placed in the Roche medium amplified very well, as seen by examination of the bands on gels, but hybridizations of PCR products placed on filters gave weak signals. This problem was traced to the presence of a high concentration of detergents in the medium, which prevented the binding of the PCR products to the filter. The problem was solved by precipitation of the specimen DNA as the first step in specimen preparation. The tests worked very well after this modification was introduced in the processing.

Comparison of three collection methods for HPV identification: Specimens collected in three different ways were compared for 189 women. The three methods were: (i) vaginal swabs placed immediately in transport medium; (ii) dry vaginal swabs, transported to Baltimore dry, by mail and placed in medium in the Baltimore laboratory; and (iii) cervical swabs placed immediately in the transport medium. The second method is the most convenient way of collection and transport of the specimens.

Table 9 shows that all three methods were satisfactory and comparable with respect to beta-globin amplification and HPV identification. The cervical wet swab appeared to be slightly more efficient than the other two methods.

Table 9
COMPARISON OF THE THREE COLLECTION METHODS FOR BETA-GLOBIN AMPLIFICATION AND HPV DIAGNOSIS

	Specimens (n = 189)	
	% Beta-globin positive	% HPV positive*
vaginal, wet	93	44
vaginal, dry	92	47
cervix, wet	95	50

*of those that are beta-globin positive

The correlation between results of the three methods is shown in Table 10. 158 of the 189 specimens (84%) were beta-globin positive in all three methods and none were negative by all three methods. With respect to HPV diagnosis, 53 specimens (28%) were positive and 77 specimens (42%) negative in all tests, for a 69% concordance of results of the three tests, for any HPV type. The type-specific concordance between assays is being currently examined in detail.

Table 10
CORRELATION BETWEEN RESULTS OF THE THREE METHODS WITH RESPECT TO BETA-GLOBIN AND HPV AMPLIFICATION

Vaginal Wet	Beta-globin	Generic HPV
	158	53
	8	10
	1	6
	8	8
	7	12
	1	6
	6	17
	0	77
	189	189

Correlates of HPV prevalence: The wet vaginal swabs from all 764 samples were tested for HPVs. Beta-globin was amplified from 720 specimens (94.3%) and HPV was identified from 279 (36.5%) of the specimens.

Table 11 gives the HPV prevalence by age, race, Pap smear diagnosis and number of sexual partners in the previous six months. The HPV prevalence declined with increasing age from a high of 60% in women under the age of 20 to 18-20% in women 29 years old or older. HPV prevalence was 35.8% in women with normal Pap smears and 89-100% in women with LGSIL or HGSIL. Higher HPV prevalence was related to greater numbers of sexual partners in the previous six months.

Table 11
HPV PREVALENCE BY SELECTED VARIABLES

		Number	Number (%) positive
Age	<20	79	47 (59.5)
	21-24	241	102 (42.3)
	25-28	182	63 (34.6)
	29-32	70	14 (20.0)
	32+	76	14 (18.4)
Race	White	238	100 (42.0)
	Black	424	145 (34.2)
	Other	57	33 (57.9)
Pap smears	Normal	628	225 (35.8)
	LGSIL	36	32 (88.9)
	HGSIL	3	3 (100.0)
	Unsatis.	51	18 (35.3)
Number of partners (last 6 months)	0	31	7 (22.6)
	1	528	192 (36.4)
	2	117	55 (47.0)
	3	23	12 (52.2%)
	4+	21	10 (47.6)

In a preliminary analysis of the HPV type distribution in SIL lesions, it was seen that the 13 cancer-associated HPV types were far more frequent in women with SIL lesions than in women with normal cytology.

Comments and Conclusions:

1. The method of obtaining a sample, which was most suitable for self-collection by women and for transportation to the laboratory, the vaginal dry swab, was comparable to other more conventional methods of specimen collection, with respect to sample adequacy and HPV diagnosis.

2. HPVs, most often cancer-associated HPVs, were recovered from nearly all of the 39 women who had the diagnosis of SIL by cytology.

3. We intend to analyze the data to determine if the following scheme may be useful in monitoring military women for cervical SIL.
    a. Obtain self-collected dry swabs from women above the age of 29;
    b. Test specimens only for 13 cancer-associated types;
    c. Arrange for HPV-positive women to attend the clinic for a diagnostic work up including Pap smear and colposcopy.

When the analyses are complete, we will be able to project how many women will need to visit the clinic under the above scheme and estimate the predictive value of HPV-positivity for SIL, in these women. We anticipate that about 10% of the women will require a clinic visit of whom about one-third will have a SIL cytology.

Conclusions

Although analyses are still ongoing, we conclude that self-administered swabs (SAS) are at least as sensitive as standard diagnostic procedures in detecting gonorrhea, chlamydia, and trichomonas infections among active duty women presenting to the Epidemiology and Disease Control Clinic at Fort Bragg. Furthermore, SAS can also be applied to the detection of type specific human papillomavirus. We also discovered that the SAS can be successfully transported in a dry stage and has equal performance in detecting gonorrhea, chlamydia and HPV organisms as that transported in standard transport medium (wet stage). The SAS and specimen stability data collected in this study indicate that this approach, namely a non-invasive, self-administered swab specimen for STD detection, has the potential to drastically improve STD screening in military women. Use of SAS diagnostics for all common STDs could revolutionize the approach to efficient and cost effective STD testing for military women in an effort to reduce the morbidity associated with these diseases. Not only could access to medical care through collecting and sending the specimen to a medical facility by mail or courier be increased, but compliance to submit to STD screening may be enhanced greatly, since a speculum examination of the vagina and cervix could be avoided, at least for the screening test.

STATEMENT OF WORK

Task 1: Proposed: Month 1-3: Hire and train Ft. Bragg Study Nurse and Study Assistant. Develop and duplicate study questionnaire.

Completed: Nurse and study assistant trained and study integrated into the regular clinic flow.

Task 2: Proposed: Months 4-18: Enroll approximately 85 active duty military women attending the Epidemiology and Disease Control (EDC) Clinic at Ft. Bragg per month into study. Federal Express self-administered swabs (SAS) and clinical specimens three times weekly to the Johns Hopkins University HIV/STD Research Laboratories. Maintain laboratory results reporting by FAX.

Completed: Enrollment began in March and continued through October 1998. Approximately 800 women were recruited, for an average of 40 women per month. Enrollment was lower than expected due to unexpected clinician shortage and decreased patient attendance. A smooth system for shipping specimens was instituted and results were Faxed in a timely fashion.

Task 3: Proposed: Months 4-18: Determine sensitivity and specificity of the SAS to detect gonorrhea, chlamydia, trichomonas, and human papilloma virus infections using PCR techniques compared to that obtained by standard diagnostic tests and selected gold standard tests performed on specimens obtained during speculum-assisted gynecologic examination.

Completed: Sensitivity and specificity analyses are being completed. Since specimens were collected until the closing month of the study, the complete data set which incorporates results from each laboratory and the EDC clinic data base is being constructed and cleaned.

Task 4: Proposed: Months 14-18: Determine the performance ease of patient-administered SAS among a subgroup of 275 women attending the EDC and consenting to study participation. Modify the collection kit and self-use instructions to improve utility.

Completed: A total of 325 women were approached and agreed to the self-collected SAS procedures. No modification of instructions was needed.

Task 5: Proposed: Months 19-20: Analyze study questionnaire to identify risk factors, reasons for EDC visit, STD prevalence rates.

Completed: As discussed for Task 3, the complete data set is being cleaned and analyzed for publication.

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Diagnosis of *Trichomonas vaginalis* Infection by PCR Using Vaginal Swab Samples

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***Trichomonas vaginalis* infection is the most prevalent nonviral sexually transmitted disease (STD) in the world. A PCR test using vaginal swab samples for the detection of *T. vaginalis* was developed to add *T. vaginalis* infection to the growing list of STDs that can be detected by DNA amplification techniques. A primer set, BTUB 9/2, was designed to target a well-conserved region in the beta-tubulin genes of *T. vaginalis*. All strains (15 of 15) of *T. vaginalis* tested were successfully detected by PCR giving a single predicted product of 112 bp in gel electrophoresis. No such targeted product was amplified with DNA from *Trichomonas tenax*, *Trichomonas gallinae*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Giardia lamblia*, *Chilomastix sulcatus*, *Dientamoeba fragilis*, and *Entamoeba histolytica*. An optimal analytical sensitivity of one *T. vaginalis* organism per PCR was achieved. Culture, performed with the Inpouch TV culture system, was examined daily with a light microscope to identify *T. vaginalis*. Twenty-three of 350 (6.6%) vaginal swab samples from women attending an army medical clinic were culture positive for *T. vaginalis*. Of these culture positive specimens, PCR detected 22 of 23 (96%) with primer set BTUB 9/2, and wet preparation detected only 12 of 23 (52%). Seventeen specimens were BTUB 9/2-PCR positive and culture negative. Ten of these discordant specimens were determined to be as true positive by PCR using primer sets TVA 5-1/6 and/or AP65 A/B, which target different regions in the *T. vaginalis* genome, and seven were determined to be false positive. The sensitivity of BTUB 9/2-PCR was 97% and the specificity was 98%. The sensitivities of culture and wet preparation were 70 and 36%, respectively. The diagnosis of *T. vaginalis* infection by PCR is a sensitive and specific method that could be incorporated into a joint strategy for the screening of multiple STDs by using molecular amplification methods.**

Trichomonas vaginalis infection is the most prevalent nonviral sexually transmitted disease in the world (18). In the United States, an estimated 3 million woman contract trichomoniasis every year (12). Symptomatic women with trichomoniasis usually complain of vaginal discharge, vulvovaginal soreness, and/or irritation. Dysuria and dyspareunia are also common (18, 21). *T. vaginalis* can be asymptomatic in 10 to 50% of women (9, 28). The organism can be recovered in 11% of men attending sexually transmitted disease (STD) clinics (13) and from 30 to 60% of male sexual partners of infected women, usually as a self-limited mild urethritis (9, 13). It can be transmitted to neonates during passage through an infected birth canal (2 to 17%), but the infection is usually asymptomatic and self limited (4). The incidence of trichomoniasis is highest in women with multiple partners and in groups with a high prevalence of other STDs (3). Whether trichomoniasis is a risk factor for human immunodeficiency virus transmission or just a marker for high-risk heterosexual activity remains unclear (15). An association of pelvic inflammatory disease, tubal infertility, and cervical cancer with previous episodes of trichomoniasis has been reported but may be explained by its association with other STDs (16, 17, 29). Complications of trichomonal vaginitis that have been reported include premature rupture of membranes, premature labor, low birth weight, and post-abortion or post-hysterectomy infection (2, 8, 9, 21, 22).

Traditionally physicians make the diagnosis based on clinical grounds, but in women, the characteristics of the vaginal discharge, including color and odor, are poor predictors of *T. vaginalis* (21, 23). Since no symptom alone or in combination is sufficient to diagnose *T. vaginalis* infection reliably, laboratory diagnosis is necessary (28). *T. vaginalis* may be identified in vaginal secretions by using a wet preparation, but this method is only 35 to 80% sensitive compared with culture (6, 14). The sensitivity of wet preparation is highly dependent on the expertise of the microscopist (9) and the prompt transport and laboratory processing of samples before the organism becomes lysed or loses motility. Other diagnostic techniques, such as fluorescent antibody (14), enzyme-linked immunosorbent assay (20), and a hybridization test (5), have been used to detect *T. vaginalis* and have had reported sensitivities between 70 and 90% (14). Culture in microaerophilic conditions is estimated to be 85 to 95% sensitive and has been considered the "gold standard" for diagnosis (9).

In this study, a PCR targeting the beta-tubulin genes of *T. vaginalis* was developed for the detection of the organism in vaginal swab samples. The targeted genes encode the amino acid sequence of beta-tubulin protein (11), a major component of the *T. vaginalis* cytoskeleton. Trichomonas PCR was compared with culture and wet preparation. Discrepant results were adjudicated by PCR using a previously described set of primers (19) and a set of primers targeting the adhesin genes of *T. vaginalis*. Since elevated pH of vaginal secretions in pa-

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                841          ------------  btub9 ------------>
btub1              agttttctgc attgataacg ------aagc tctttacgat atctgcttcc gtacactcaa gctcacaaca
btub2              .......... .......... ------.... a......... ..t....... .......... ..........
btub3              ...c..t... .......... ------.... .......... .......... .......... ..........
G. lamblia         g..c...... ..c..c.... ------.g.. c..c.....c .......... .c..g..... ......gtgc
T. brucei          gtcga.g... .....c.... ------.... a..g..... ..t....... .c..c..g.. a..g......
T. cruzi           .tcga.g..t .....c..t. ------.... c..g..... ..t..t..t. ....gt.g.. a..g..g..t
T. gondii          ...ccaggtg ..c..c.... ------.g.. c..a.....c .......... .a.....g.. ...g..c..g
H. sapiens         gacc.at... .....c.... ------.g.. c..c..t... .......... .c..t..g.g .g.g..c...
P. falciparum      t.a.gaagtt caa.t..t.. ataatg.... .t.a..t..c ..a..t..ta .g..t..t.. at.a......

                905                                          <--------- btub2 ----------
btub1              ccaacatacg gcgatcttaa ccaccttgtt tccatggtta tgtccggcac aacatgcgct ctccgcttcc
btub2              .......... .......... .......... .......... .......... .......... ......t.a ...a.a....
btub3              .......... .......... .......... .......... .......... .......... .......... ..........
G. lamblia         ..c..c.... .a..c..c.. ......c..c ..gc.c..c. ........tg c..ga..tgc ..........
T. brucei          .....g.t.. ....c..g.. ....t.g..g ..tgct...g ........gt c..c...tgc ..g.......
T. cruzi           .....g.t.. .t.....g.. ....t.g..g ...gc...gg .t......gt g..c...tgc ..g.......
T. gondii          ..g..g..t. ....c..g.. ...tt.g..c ...gca.cc. ..ag...tgt g..c...tgt ..........
H. sapiens         ..c..c..t. .g...acc.. ..t...c..g .aagacacc. ..gagt.tgt c..cac.tgc ..........
P. falciparum      ........t. .a...t.a.. t........a ..agct.ca. ....a..tgt ...c..tt.g t.aa.a..t.
```

FIG. 1. PCR primers BTUB 9 and BTUB 2 for the detection of *T. vaginalis* with the beta-tubulin genes. Primers were selected from well-conserved and specific regions of the genes (*btub1*, *-2*, and *-3*). Sequences of the beta-tubulin genes of *T. vaginalis* were compared to the beta-tubulin gene sequences of humans and to those of other pathogens. A dot indicates the same base, and a letter indicates a different base compared to the *T. vaginalis btub1* gene sequence.

tients infected with *T. vaginalis* has been reported (21), its utility as a predictor was also explored in this study.

MATERIALS AND METHODS

***T. vaginalis* strains.** One strain from the American Type Culture Collection (Manassas, Va.) (ATCC SF-314 030001) and 14 strains of *T. vaginalis* (designated A to N) isolated by culture of vaginal secretions from patients attending an STD clinic were used to assess the sensitivity of the PCR primer sets. The specificity was evaluated by testing other related *Trichomonas* spp., flagellates, amoebae, or cervicovaginal pathogens (*Trichomonas tenax* ATCC 30207, *Trichomonas gallinae* ATCC 30002, *Giardia lamblia* ATCC SF-741 30888, *Chilomastix sulcatus* ATCC 50562, *Dientamoeba fragilis* ATCC 30948, *Entamoeba histolytica* ATCC SF-31-90015, *Chlamydia trachomatis* serovar E ATCC VR 3488, and *Neisseria gonorrhoeae* ATCC 19424).

DNA from *T. vaginalis* or the other microorganisms mentioned above was prepared from cultures by using the Chelex method (25). Mixtures of 50 μl of cultures with 200 μl of a 5% suspension of chelating resin (Chelex 100; Sigma, St. Louis, Mo.) in Tris buffer (0.01 M [pH 8.0]) were incubated at 56°C for 15 to 30 min. Preparations were mixed gently and then boiled for 8 to 10 min. After mixing again, preparations were centrifuged (12,000 × *g*) for 1 min in a microcentrifuge and stored at −70°C.

The analytical sensitivity was assayed with one clinical strain of *T. vaginalis* (strain M). Twofold dilutions of trichomonas organisms in culture media were initially prepared with 128 organisms (counted with a hemocytometer counting chamber) per PCR amplification, but the optimal analytical sensitivity of one organism per PCR amplification was achieved. Each dilution was processed separately by using the Chelex method to extract the DNA.

Clinical specimens. Informed consent was obtained from all study participants. Before insertion of the speculum, clinicians collected vaginal swab samples (n = 350) from women attending the Epidemiology and Disease Control Clinic at the Womack Army Medical Center, Fort Bragg, N.C., from March to December 1997. The median age of the sampled population was 24 (range, 18 to 47), and 30% were Caucasian, 64% were African American, and 6% were of other races.

Vaginal swab samples were placed in 1 ml of a commercial PCR transport medium (AMPLICOR; Roche Diagnostic Systems, Branchburg, N.J.) and kept at 4°C until arrival at the laboratory within 4 days of collection. An equal volume of specimen diluent (AMPLICOR) was added to the sample, and the preparation was mixed, incubated at room temperature for 10 min, and stored at −70°C until tested.

A second vaginal swab sample was obtained after the insertion of the speculum. It was immediately touched to a glass slide together with a drop of normal saline for the microscopic (×100) wet examination of trichomonas in vaginal fluid. After the wet preparation was made, the swab was immediately inoculated into the Inpouch TV culture system (Biomed Diagnostics, Santa Clara, Calif.) (6). The Inpouch culture bag was sealed, incubated at 30°C, and examined daily with a light microscope to identify *T. vaginalis*. The pH of vaginal secretions was measured by using pH test strips (Sigma).

PCR primers. A set of primers targeting a conserved region of the beta-tubulin genes of *T. vaginalis* (*btub1*, *-2*, and *-3*) was designed, synthesized, and tested. The sequences were as follows: for BTUB 9, 5′ CAT TGA TAA CGA AGC TCT TTA CGA T 3′ (positions 850 to 874); and for BTUB 2, 5′ GCA TGT TGT GCC GGA CAT AAC CAT 3′ (positions 961 to 938).

The DNA sequences for the primer set BTUB 9/2 were designed to target conserved regions of the three beta-tubulin genes of *T. vaginalis* to improve the analytical sensitivity (GenBank accession numbers: *btub1*, L05468; *btub2*, L05469; and *btub3*, L05470). In addition, to improve specificity, these DNA sequences were selected from the regions of the *T. vaginalis* beta-tubulin genes that differed substantially from the beta-tubulin gene sequences of humans and other microorganisms. (GenBank accession numbers: *G. lamblia*, X06748; *Trypanosoma brucei* subsp. *rhodesiense*, K02836; *Trypanosoma cruzi*, M97956; *Toxoplasma gondii*, M20025; *Homo sapiens*, V00598 J00317; and *Plasmodium falciparum*, G9981) (Fig. 1).

Discrepant results between the BTUB 9/2 PCR, wet preparation, and culture were adjudicated by PCR with primer set TVA 5/6, which has been previously described (19), and with primer set AP65 A/B, which is designed to target the adhesin genes of *T. vaginalis* (1). Primer TVA 5 was slightly modified by removing four bases at the 5′ end to avoid primer dimer formation and by including four more bases of the targeted sequence (102-bp clone A6p) at the 3′ end (our TVA 5-1), as follows: for TVA 5, 5′ GATC ATG TTC TAT CTT TTC A 3′ (positions 1 to 20); for TVA 5-1, 5′ ATG TTC TAT CTT TTC A TTGT 3′ (positions 5 to 24); and for TVA 6, 5′ GAT CAC CAC CTT AGT TTA CA 3′ (positions 102 to 83). Primer set AP65 A/B was designed to target a conserved region in the ap65 adhesin genes (GenBank accession numbers U18346 and U18347). Genes *ap65-1* and *-2* encode the AP65 protein that mediates *T. vaginalis* cytoadherence to the vaginal epithelium (1). The sequences of the primers were as follows: for AP65 A, 5′ GAT TCC TCT TCA CAC ACC CAC CAG 3′ (positions 304 to 327); and for AP65 B, 5′ AAT ACG GCC AGC ATC TGT AAC GAC 3′ (positions 512 to 489).

PCR. PCR was performed with 10 μl of cultures (including all *T. vaginalis* strains and the other microorganisms mentioned above) processed by the Chelex method, or with 50 μl of vaginal swab samples processed with AMPLICOR specimen buffers. PCRs were performed in an automated thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). The final reaction mixture (100 μl) contained 25 pmol of each primer, 2.5 mM deoxynucleoside triphosphate, 1× PCR buffer (10 mM Tris HCl [pH 8.4], 50 mM KCl), and 2 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer Cetus) overlaid with 2 drops of mineral oil. Since 1.0 mM $MgCl_2$ is a component of the AMPLICOR specimen buffers, 1.5 mM $MgCl_2$ was added to the reaction mixture; otherwise, 2.5 mM $MgCl_2$ was used when cultures of microorganisms processed with Chelex were tested.

A touchdown method for thermal cycling was used. Cycling times were 75 s at 95°C followed by 60 cycles of denaturation temperature 95°C for 45 s, annealing temperature beginning at 62°C and ending at 52°C for 45 s, and extension temperature of 72°C for 1 min. The annealing temperature was lowered one degree every four cycles until reaching 52°C, and this annealing temperature was then kept until the end of the cycling process.

To avoid product carryover, PCRs were set up in a room separate from all activities involving amplified target sequences, including thermocycling areas, PCR product storage, and gel electrophoresis. A separate set of pipetting devices was devoted to the setup of PCRs, and aerosol barrier pipette tips were used. Negative controls, including uninoculated transport media, were used through-

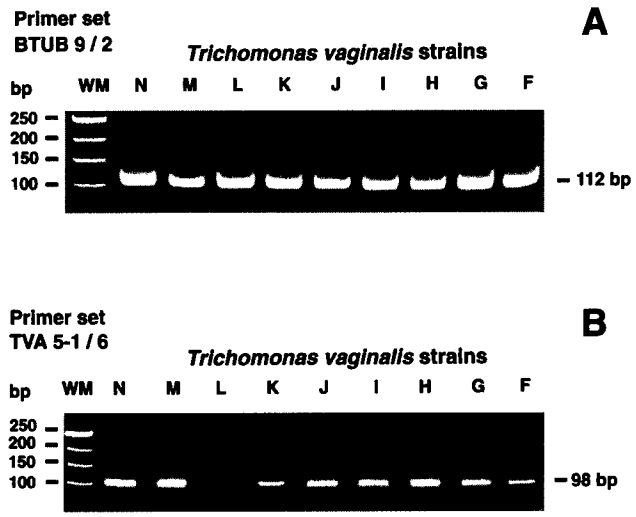

FIG. 2. Detection of *T. vaginalis* strains F to N by PCR with primer sets BTUB 9/2 (A) and primer set TVA 5-1/6 (B). Strains F to N are representative of 14 strains of *T. vaginalis* (designated A to N) isolated from vaginal secretions of patients attending a city STD clinic in Baltimore, Md.

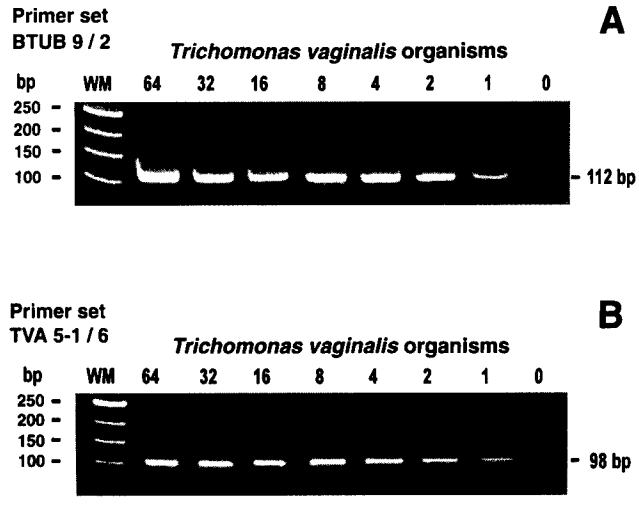

FIG. 3. Analytical sensitivity testing of *T. vaginalis* PCR with primer set BTUB 9/2 (A) and primer set TVA 5-1/6 (B). Twofold dilutions of strain M of *T. vaginalis* were processed separately to extract the DNA and were tested by PCR.

out the specimen preparation and PCR process. A low copy number (n = 50/PCR) of trichomonas as positive control was included in every PCR run.

Detection of amplified targets. The primer set BTUB 9/2 was designed to amplify a DNA product of 112 bp from the three beta-tubulin genes (Fig. 1). Primer set TVA 5-1/6 amplified a DNA product of 98 bp from a segment of the *T. vaginalis* genome (102-bp clone A6p). Primer set AP65 A/B amplified a DNA product of 209 bp from both *ap65-1* and *-2* adhesin genes of *T. vaginalis* (sequence numbers 304 to 512).

Twenty microliters of amplified product was electrophoresed at 120 V in 12% polyacrylamide gels in Tris-borate-EDTA buffer and stained with ethidium bromide (0.01 mM). The sizes of the amplified products were assessed by comparison with a commercial 50-bp weight marker (XIII; Boehringer Mannheim, Indianapolis, Ind.).

Adjudication of discrepant results and statistical analysis. Discrepant specimens (those found to be culture negative and PCR positive) were determined to be *T. vaginalis* positive by performing PCR with other primer sets (TVA 5-1/6 and AP65 A/B). The gold standard was considered to be either culture positivity or PCR positivity with primer set BTUB 9/2 and at least one other primer set (TVA 5-1/6 or AP65 A/B). Sensitivity and specificity were calculated by using the defined gold standard. Confidence intervals for sensitivity and specificity were determined based on a normal approximation to the binomial distribution. Agreement between PCR and culture was assessed by using the kappa test (7).

RESULTS

Primer set BTUB 9/2 amplified the predicted 112-bp product in all 15 *T. vaginalis* strains tested. The analytical sensitivity of PCR with primer set BTUB 9/2 on the twofold dilutions of *T. vaginalis* organisms was the amplification of the DNA of one organism per PCR (see Fig. 3). No targeted PCR products were amplified when DNAs from other vaginal pathogens or protozoa were tested with the BTUB 9/2 primer set. However, a slightly bigger product (125 bp) was obtained (in gel electrophoresis) when DNA from the oral *T. tenax* was tested.

PCR primer sets TVA 5-1/6 and AP65 A/B, which were used to resolve discrepant results in our clinical study, amplified the predicted 98-bp product in 14 of 15 strains of *T. vaginalis* and the predicted 209-bp product in 15 of 15 strains of *T. vaginalis*, respectively. Neither primer set TVA 5-1/6 (Fig. 2) nor primer set TVA 5/6 (not modified) amplified one particular clinical strain of *T. vaginalis* (strain L). None of the other vaginal pathogens or protozoa tested was detected by primer sets TVA 5-1/6 or AP65 A/B. The DNA corresponding to one copy of *T. vaginalis* (strain M) was amplified by both sets of primers TVA 5-1/6 (Fig. 3) and AP65 A/B (data not shown).

Twenty-three of 350 vaginal swab samples (6.6%) were culture positive for *T. vaginalis*. Wet preparation detected 52% (12 of 23), and BTUB 9/2 PCR detected 96% (22 of 23) of these positive cultures. Seventeen specimens were BTUB 9/2 PCR positive and culture negative. Ten of these discrepant specimens were determined to be true positives by PCR with primer sets TVA 5-1/6 and/or AP65 A/B targeting different regions in the *T. vaginalis* genome, and seven could not be confirmed as positives and were considered false positives (Table 1). Primer set TVA5-1/6 detected 61% (13 of 23) of specimens positive by culture. Primer set AP65 A/B detected 4 of 9 (44%) positive cultures that were not detected by PCR with TVA 5-1/6, and 6 of 10 (60%) negative cultures detected positive by two or more PCR primer sets (Table 1). Three specimens that were found to be positive by wet preparation could not be confirmed positive by culture or PCR with any of the primer sets tested and were deemed false positives.

After resolving discrepant results that were culture negative and PCR positive, the sensitivity of PCR with the primer set BTUB 9/2 was 97% (32 of 33; 95% confidence interval [CI], 95.2 to 98.8), and the specificity was 98% (310 of 317; 95% CI, 96.3 to 99.3) (Table 2). The sensitivity of culture was 70% (23

TABLE 1. Resolution of discrepant results between culture and PCR for the detection of *T. vaginalis* in 350 vaginal swab samples

No. of samples	Result by culture[a]	Result with PCR primer set:		
		BTUB 9/2	TVA 5-1/6	AP65 A/B
14	+	+	+	NT[b]
4	+	+	−	+
4	+	+	−	−
1	+	−	−	−
5	−	+	+	+
4	−	+	+	−
1	−	+	−	+
7	−	+	−	−
310	−	−	−	NT

[a] As determined the Trichomonas Inpouch TV culture system (Biomed Diagnostics, Santa Clara, Calif.).
[b] NT, not tested.

TABLE 2. Comparison of PCR performed with primer set BTUB 9/2 and TVA 5-1/6, culture, and wet preparation for the detection of *T. vaginalis* in 350 vaginal swab samples

Method	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
PCR				
With BTUB 9/2	97.0	97.8	82.1	99.7
With TVA 5-1/2	69.7	100	100	96.9
Culture[a]	69.7	100	100	96.9
Wet preparation	36.4	99.1	80.0	93.7

[a] Values obtained with the Trichomonas Inpouch TV culture system.

of 33; 95% CI, 64.9 to 74.5) and the specificity was 100% (317 of 317). The sensitivity of wet preparation was 36% (12 of 33; 95% CI, 31.3 to 41.4) and the specificity was 99% (314 of 317; 95% CI, 98 to 100). Although culture and the PCR with primer set TVA 5-1/6 had the same sensitivity and specificity (Table 2), their agreement was only moderate ($\kappa = 0.58$). In comparison, there was substantial agreement between culture and PCR with primer set BTUB 9/2 ($\kappa = 0.78$). The prevalence of *T. vaginalis* in this STD population was 9.4% (33 of 350) after the adjudication of discrepant results.

A similar proportion of confirmed trichomonas-positive patients was symptomatic (24 of 33 [72.7%]) compared to patients who were negative for trichomonas (230/317 [72.6%]) ($P = 0.9$). Sixty-seven percent (22 of 33) of confirmed *T. vaginalis*-positive patients had vaginal secretions of pH of >4.5 compared to 43% (136 of 317) of negative patients ($P = 0.009$). The odds ratio of having a trichomonas infection was 2.7 (95% CI, 1.3 to 5.6) when pH was >4.5. In this study, treatment for trichomoniasis was provided according to clinic standard methods on the basis of wet preparation results. In a retrospective analysis, 52% (17 of 33) of patients with confirmed trichomonas infections received appropriate treatment with metronidazole at the clinic site, five of them because bacterial vaginosis was suspected. In addition, no diagnosis or treatment was given to 15% (5 of 33) of infected patients even though three had genitourinary symptoms (Table 3).

DISCUSSION

Trichomonas PCR with primer set BTUB 9/2 was 97% sensitive and 98% specific. It was more sensitive than wet preparation or culture. One culture-positive specimen could not be detected by any of the three PCR primer sets and was assumed to be a true positive by definition. The number of confirmed *T. vaginalis*-positive samples was increased by 39% by PCR compared to culture (32 of 350 [9.1%] versus 23 of 350 [6.6%]). PCR with primer set BTUB 9/2 for the detection of *T. vaginalis* had good analytical sensitivity and was able to amplify one trichomonas organism per PCR. The predicted DNA product (112 bp) in the targeted beta-tubulin gene was amplified with all *T. vaginalis* strains tested (15 of 15). The analytical specificity of primer set BTUB 9/2 was optimal, since no targeted DNA products were detected with other protozoa or vaginal pathogens.

In the *T. vaginalis* genome, there are several copies of the three genes encoding beta-tubulin proteins (beta-tubulin 1, 2, and 3) (11). Primer set BTUB 9/2 was designed to target a well-conserved region in all three beta-tubulin genes, thus improving sensitivity because of increased number of DNA target copies available for amplification. This region appears to be moderately conserved across other trichomonas species, because a slightly bigger PCR product of 125 bp was amplified by primer set BTUB 9/2 when DNA from the oral *T. tenax* was tested but not with DNA from *T. gallinae*. Although the 125-bp product detected with DNA of *T. gallinae* is similar in size to the product of *T. vaginalis* (112 bp), which could be difficult to differentiate by standard gel electrophoresis, specificity should not be affected since each *Trichomonas* species appears to be limited to its specific site of colonization (*T. tenax* inhabits the mouth and *T. vaginalis* only the genital and urinary tracts) (13).

The specificity of the primers depends on the annealing temperature, with higher temperatures favoring higher specificity. The use of a touchdown protocol increased the specificity of the PCR by favoring the amplification of targeted copies of DNA amplified during early cycles at higher annealing temperatures and eliminating spurious products. The DNA polymerase used (AmpliTaq Gold) is not active until heated to 95°C, thus simulating a hot-start PCR technique. The use of this enzyme also avoided erroneous amplification of DNA products due to nonspecific annealing of primers at lower temperatures.

In our study, PCR with primer set TVA 5-1/6 detected 61% (14 of 23) of culture-positive specimens. In previous studies, when compared with culture, PCR with primer set TVA 5/6 detected 90% (44 of 49) of culture-positive distal vaginal swab samples from an STD clinic in Pittsburgh, Pa. (10), and detected 91% (41 of 45) of culture-positive tampon specimens collected from a population of the Torres Strait Island (24). The target sequence of primer set TVA 5/6 is the full length of the 102-bp A6p clone isolated by Riley et al. from the *T. vaginalis* genome with the restriction enzyme *Sau*3A (recognition sequence, ↓ GATC [the arrow indicates the cleavage site]) (19). Since both TVA5 and TVA6 primers included this recognition sequence at the 5′ end, we designed the primer TVA 5-1, which excluded the GATC sequence, to avoid the formation of primer dimers during amplification. This slight modification is unlikely to explain the difference in the proportion of positive cultures detected by PCR with primer set TVA 5-1/6 compared to previous studies with primer set TVA 5/6 (not modified). A possible explanation for this difference is that a genetically more diverse group of *T. vaginalis* strains were represented in the population of our study, which differed in the targeted region of primer set TVA 5/6 and were not detected. This conclusion is supported by the failure of primer sets TVA 5-1/6 or TVA 5/6 to detect *T. vaginalis* strain L.

The Chelex method for DNA preparation from cultures was simple, inexpensive, and easy to perform and prevented contamination as a result of manipulation of specimens used in other DNA preparation methods. As chelating resin, Chelex

TABLE 3. Clinical diagnosis and treatment received by patients with PCR-confirmed infections with trichomonas[a]

Clinical diagnosis	No. of patients	Treatment	No. of genitourinary symptoms
Vaginitis (trichomoniasis)	12	Metronidazole	10
None	5	None	3
Vaginitis (bacterial vaginosis)	5	Metronidazole	4
Vaginitis (unspecified)	5	Doxycycline	3
Vulvovaginal candidiasis	2	Antifungal	1
Genital herpes	2	Acyclovir	2
Chlamydia	1	Doxycycline	1
Condylomata acuminata	1	Not specified	1

[a] $n = 33$. Treatment for trichomoniasis at the clinic was given on the basis of wet preparation results.

100 has been used to extract heavy metals from solutions and, when used for DNA extraction, may also eliminate PCR inhibitors present in the samples and/or prevent the disruption of the genomic DNA as suggested by Walsh et al. (25).

Vaginal swab samples for the PCR screening of *T. vaginalis* are adequate and are comparable to posterior vaginal vault specimens collected with a speculum during a pelvic examination (27). In our study, the vaginal swab samples were collected by the clinicians, but self-administered vaginal swab samples, which are practical, rapid, and easy to obtain, can also be used for screening. Other studies have demonstrated that self-administered vaginal swab samples are comparable to physician-administered samples for the PCR detection of trichomonal infection (26).

An increased pH of the vaginal secretions in patients infected with *T. vaginalis* has been previously reported (21). In our study, two-thirds of the patients with confirmed positive *T. vaginalis* infection had vaginal secretion pHs of >4.5. Nevertheless, these patients represented only 16% of the total number of patients with elevated pHs, making pH an inadequate predictor for trichomonas infection.

Retrospectively, only 36% of patients with confirmed *T. vaginalis* infection were diagnosed by wet preparation, only 52% received appropriate treatment with metronidazole at the clinic, and no clinical diagnosis or treatment was given to 15% of the infected patients. These findings suggest the need for a more accurate diagnostic test for trichomonas, such as PCR, to prevent complications, transmission to sexual partners, and asymptomatic carriers, and to decrease possible transmission of HIV (2, 8, 9, 15, 21, 22).

Although wet preparation had minimal cost, its sensitivity is highly dependent on the expertise of the microscopist (9), prompt transport, and laboratory processing before the organism lyses or loses motility. In our study, even when the wet preparation was performed at the collection site, its sensitivity (36%) was suboptimal compared to PCR. Culture had a better sensitivity (70%) than wet mount examination but required more time for laboratory turnaround since cultures are held for 1 week. PCR results are available in 2 to 3 days and provided the highest sensitivity. The cost of PCR testing comes mainly from the cost of reagents, which is about two dollars per specimen, approximately equivalent to the cost of an Inpouch TV culture bag. The technician time is probably equivalent since cultures require daily examination by light microscopy. Although trichomonas PCR requires more technical skill, molecular amplification techniques are currently in use in many laboratories for the detection of *C. trachomatis* and *N. gonorrhoeae* infections. Thus, trichomonas PCR could easily be incorporated into the work flow of other diagnostic amplification procedures.

Vaginal swab samples for trichomonas PCR were transported in and processed with AMPLICOR specimen buffers, which is the transport and processing method for the *C. trachomatis-N. gonorrhoeae* PCR test (AMPLICOR, Roche Diagnostic System) currently in research use in our laboratory. Preliminary results indicate that 2 sucrose phosphate, which is a common chlamydia culture transport medium, is also an adequate transport media for this trichomonas PCR method, as well as for the *C. trachomatis-N. gonorrhoeae* PCR test, as suggested by the manufacturer. Thus, specimens transported in 2 sucrose phosphate media can be subsequently processed by the Chelex method. Incorporating PCR with BTUB 9/2 for *T. vaginalis* into the routine laboratory diagnostic methods using the same processed sample used for the PCR detection of *C. trachomatis* and *N. gonorrhoeae* could be a cost-effective strategy when screening programs for multiple STDs are implemented.

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PHYLIS M. RINEHART
Deputy Chief of Staff for
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